STUDIES ON CELL-MEDIATED IMMUNITY TO LYMPHOCYTIC CHORIOMENINGITIS VIRUS IN MICE

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In a recent review, Volkert and Lundstedt have discussed the available data which indicated the existence of an independent cell-mediated immunity to the LCM virus in mice (1). It was concluded that this immunity was provoked during the acute phase of the infection and that it was probably the decisive factor for the outcome of the infection. During the next 2 yr other authors published experimental reports which supported this assumption. Tosolini and Mims employed the footpad test in acutely infected mice and were able to demonstrate the time-course of the development of a D-type hypersensibility reaction (2). Moreover, the original work by Lundstedt which indicated a cytotoxic effect of immune lymphocytes on LCM virus-infected target cells (3) was confirmed by Holtermann and Majde (4). Finally, Wright et al. have employed the Hellström method in the same kind of experiments and have published data which might indicate that the cytotoxic effect of lymphocytes from immune donors can be suppressed by different kinds of immunosuppressive treatments (5).

In spite of the fact that all parties concerned seem to agree on the importance of the cell-mediated immunity to LCM virus, very little is as yet known about the mechanism involved in this immune reaction. The main reason for this is the lack of a reliable and reproducible in vitro method for measuring the cell-mediated immunity provoked by an LCM viral infection. A method of this type has been developed in our laboratory and it is the purpose of this paper to present the results obtained concerning the cell-mediated immunity to the LCM virus in mice.

Materials and Methods

Animals.—For virus titration purposes, ordinary Swiss mice weighing 12–14 g were used. For the experiments proper, strictly inbred C3H mice kept and bred in this laboratory were used throughout the study.

Persistent tolerant virus carriers (virus carriers) were produced by inoculating newborn mice within the first 18 h of life with approximately 10^3 LD_{50} of the virus intraperitoneally (i.p.).[1] As previously described (6), this treatment results in a state of immunological tolerance, and the mice will carry virus in high titers in blood and organs throughout life.

Virus.—The LCM virus strain used was originally obtained from Dr. Traub (Tübingen,

[1] *Abbreviations used in this paper:* CF, complement fixation; CI, cytotoxic index; FBS, foetal bovine serum; i.p., intraperitoneally; MEM, minimal essential medium; PBS, phosphate-buffered saline.

Germany). For infection of mice, a virus suspension produced from virus carrier spleens was used. The spleens were removed aseptically, ground in a mortar with sterile sea sand, and suspended in 1 ml of phosphate-buffered saline (PBS) with 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin solution (P/S) per spleen. The virus preparation was kept in ampoules at −70°C, and its titer varied from $10^{4.5}$ to $10^{5.3}$ LD_{10}/0.03 ml.

For infection of the L cells, which served as target cells in the cytotoxic reaction, virus propagated in tissue culture was employed. For this purpose monolayers of L cells in Roux bottles were infected in the conventional manner for 60–90 min. After the infection, 40 ml of minimal essential medium (MEM) with 5% FBS and 3% bicarbonate was added, and 48 h later the tissue culture medium was harvested. It was ampouled and stored at −70°C; the titer of this virus preparation ranged from $10^{5.3}$ to $10^{7.0}$ LD_{50}/0.03 ml.

The virus titrations were all carried out using intracerebral injections of serial 10-fold dilutions in ordinary Swiss mice. The titers were calculated by the method of Kärber (7) and expressed as LD_{50}/0.03 ml.

Complement Fixation Test (CF Test).—The standard procedures used have been described in a previous paper (8). The method was altered only in that Sever's microtechnique (9) was employed.

The Cytotoxic Test.—The cytotoxic test is based on a combination of a ^{51}Cr release method for measuring cell-mediated allograft immunity in vitro, described by Brunner et al. (10), and the Hellström test for lymphocyte-mediated cellular immunity (11). It was carried out as follows:

Lymphocyte suspensions: Spleen, superficial inguinal, and axillary lymph nodes were removed aseptically, cut into small pieces, and pressed through a stainless steel mesh. This procedure and the following two washings were carried out in Hanks' balanced salt solution with 0.5% lactalbumin. Finally, the cells were resuspended in MEM with 15% FBS and 3% bicarbonate to a concentration of 4.4×10^6 per ml.

L cells: 3–5-day old monolayer cultures of L cells were trypsinized and a suspension containing 1.7×10^5 cells/ml was prepared in MEM with 0.5% FBS and 3% bicarbonate. LCM virus to a multiplicity of 10–40 LD_{50} per L cell was added and the cells were kept in suspension for 90 min at 37°C, at which point MEM with 3.8% FBS and 3% bicarbonate was added to give a final concentration of 0.16×10^6 L cells/ml, and the infection was continued in suspension for further 20 h. Uninfected L cells for controls were treated in exactly the same way except that medium was added instead of virus.

Fluorescence staining: The efficiency of the infection of the L cells was checked by the direct fluorescent antibody-staining technique, as described previously (12). When the methods described are used, about 90% of the cells are infected.

Labeling: On the day of the assay, infected and uninfected L cells were counted and suspensions of each type of cells, containing 1×10^6 cells/ml, were made up in MEM with 15% FBS and 3% bicarbonate. After the adding of chromium-51 (as sodium chromate, ISOTOP service, Aktiebolaget Atomenergi, Sweden), to give 10 μCi/10^6 L cells, the cells were labeled at 37°C with magnetic stirring for 45 min. After two washings the cells were resuspended in MEM with 15% FBS and kept agitated at 37°C for 30 min. After another two washings and resuspension to a final concentration of 1.75×10^5 per ml, the cells were distributed in plastic Petri dishes (50 x 10 mm, A/S Nunc, Roskilde, Denmark), each dish receiving 4.0 ml of the cell suspension.

The reaction mixtures: After the L cells were plated the Petri dishes were placed horizontally at 37°C in a humid atmosphere containing 15–20% carbon dioxide for 60–120 min. At this point 4 ml of the final lymphocyte suspension was added, so that the ratio between target cells and the lymphocytes was 1:25, and the Petri dishes were incubated under the conditions mentioned above. All the suspensions of lymphocytes assayed were tested at least in triplicate. After 18 h, samples from each Petri dish were centrifuged and 0.400 ml of the

supernatant was transferred to plastic counting vials. Scintillation liquid was added and the amount of radioisotope was determined as counts per minute in a Beckman scintillation counter type LS-250 (Beckman Instruments, Inc., Fullerton, Calif.).

The degree of ^{51}Cr release, and the target cell destruction, was expressed as a cytotoxic index (CI) which reflects the ratio between the actual amounts of ^{51}Cr released and the total amounts incorporated. The amounts of radioisotope were measured as counts per minute and for the calculation the following fraction was used (10):

$$CI = \frac{(^{51}Cr \text{ release in the presence of immune lymphoid cells}) - (^{51}Cr \text{ release in the presence of normal lymphoid cells})}{(\text{Total } ^{51}Cr \text{ incorporated}) - (^{51}Cr \text{ release in the presence of normal lymphocytes})} \cdot 100.$$

Test for the Enhancing Antibodies.—Blood from virus carriers and from normal mice was obtained by opening the thoracic cavity and cutting the vessels at the base of the heart in mice immediately after death from inhalation of chloroform. The blood was left overnight to coagulate and then centrifuged to obtain the serum. The serum was inactivated at 56°C for 30 min.

When the labeled L cells had fixed to the bottom of the Petri dishes the medium was discarded and 0.5 ml of the serum, diluted 1:6 in PBS pH 7, from either virus carriers or normal mice was added to the dishes. The cells were incubated at 37°C in a CO_2 atmosphere and manually tilted every 5–10 min for 45 min. After the serum had been discarded, 4 ml of the lymphocyte suspension in MEM without FBS was added before incubation for another 45 min. Thereafter, 4 ml of MEM with 30% FBS was added to each dish, and the rest of the test was carried out as described above.

RESULTS

The Cell-Mediated Immunity during the Acute Infection.—The time-course of the cell-mediated immunity provoked by the LCM virus during an acute infection in adult mice was determined in four experiments. Each of these experiments was designed as follows:

Groups of six mice were inoculated i.p. with 10^3 LD_{50} LCM virus at various intervals before the assay was performed. On the day of the experiment all the mice were killed and suspensions of pooled lymphocytes from each group of animals were prepared. The cytotoxic activity of all the cell suspensions was then tested simultaneously on target cells and in culture media from the same batches.

The results from one of these experiments are recorded in Fig. 1 and the data from all four experiments are summarized in Table I. It appears that the cytotoxic activity is already detectable a few days after infection. It then increases rapidly, reaching a maximum on about day 9, and thereafter declines equally steeply. At the end of the 2nd wk after infection the cytotoxic activity is already reduced to less than half of its previous maximum. During the 3rd wk the decline is more moderate, and hereafter the cellular immunity is hardly detectable.

As shown in Table I the cell-mediated immunity provoked during the acute LCM virus infection follows roughly the same course in all four experiments.

In order to compare this cellular immunity with the humoral response to the virus, different groups of six to eight adult mice were tested individually for the presence of CF antibodies at different times after the inoculation of 10^3 LD_{50} LCM virus i.p. The data obtained are recorded in Fig. 2. In agreement with the results presented previously by other workers and with numerous

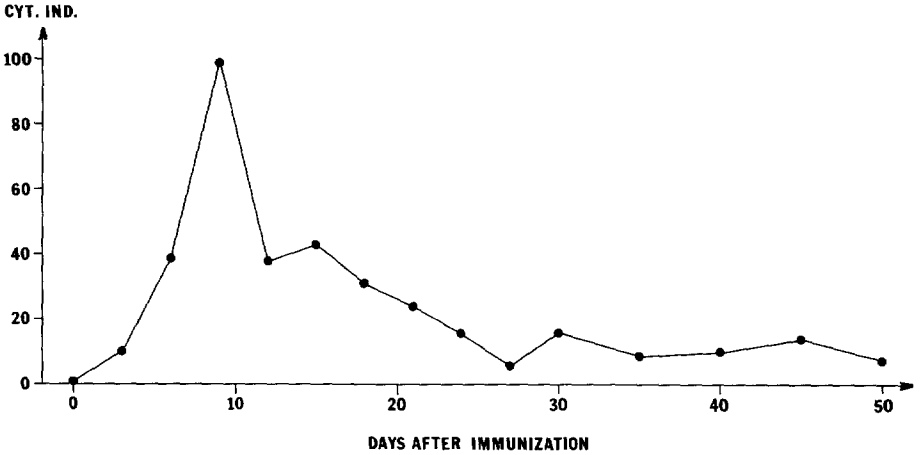

FIG. 1. The cell-mediated immune response to LCM virus in groups of normal adult C3H mice after intraperitoneal injection of 10^3 LD_{50} of LCM virus on day 0. The magnitude of the response is expressed as the cytotoxic indices (CYT. IND.) of the lymphoid cells from the different groups of mice.

TABLE I

The Time-Course of the Cell-Mediated Immunity after i.p. Inoculation of 1,000 LD_{50} LCM Virus

Exp. no.	CI on day no.								
	0	2-3	5-6	9	12	15-16	18	21	24
1	0	3	64	102	61	25	28	14	
2	0	10	39	99	38	43	31	24	16
3	0	0	0	100	100	66	40		18
4	0	0	0	76	45	6	0		0

experimental data from this laboratory, it appears that the CF antibodies are produced slowly, and that they do not reach measurable titers before day 9 or 10 after the infection. From then on the titer rises steeply, reaching a maximum at the end of the 2nd wk and then remaining constant for a long time. The development of antibodies is consequently greatly delayed compared with the cell-mediated immune reaction, and has a strikingly different time-course.

In an attempt to correlate the viremia with the time-courses of the two differ-

ent kinds of immune response various groups of six to eight adult mice were also tested for blood virus titers at different times after the i.p. injection of 10^3 LD_{50} LCM virus. Several experiments of this kind were carried out. The data obtained agree well with one another and the results from two experiments are shown in Figs. 2 and 3, which compare the blood virus titer with humoral and cellular immunity, respectively. It is evident that there is a close accordance between the graphs showing the cell-mediated immunity and the blood virus titers. However, as could be expected, in the period immediately after infection

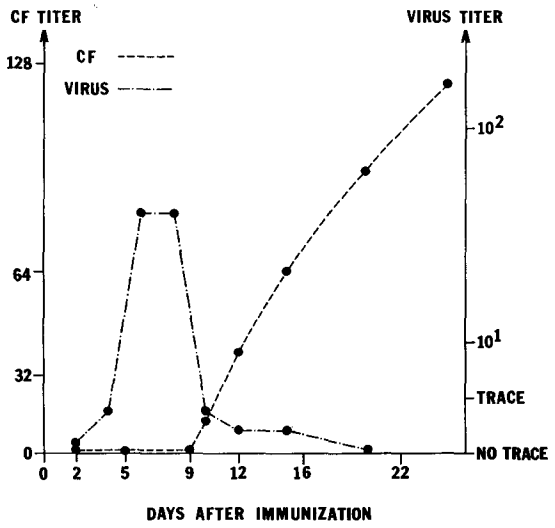

FIG. 2. Complement-fixing antibody titers and blood virus titers in normal adult C3H mice after intraperitoneal injection of 10^3 LD_{50} of LCM virus on day 0. Complement-fixing antibodies (CF TITER) and blood virus titers (VIRUS) are mean titers of six mice tested with intervals throughout the period of experimentation. (NO TRACE: virus titer $\leqq 10^{0.5}$.)

the graph showing the time-course of the blood virus titer is 3–4 days ahead of that depicting the cell-mediated immunity, while both of them decline to very low levels at the same time. This is in contrast to the humoral immunity. As can be seen from Fig. 2, the CF antibodies are just reaching detectable concentrations when practically all the virus has disappeared from the blood.

Cell-Mediated Immunity in Adoptively Immunized Virus Carriers.—Volkert, Larsen, and Pfau (8) have demonstrated that LCM virus carrier mice develop an unusually strong humoral immune reaction accompanied by a clearing of the virus from the blood and organs when the animals are transplanted with lymphoid cells from immune donors. In view of these findings it seemed interesting to determine the cell-mediated immunity during adoptive immunization of virus carrier mice.

As donors were chosen female C3H mice which had been immunized to the virus by nursing infected babies 6–8 wk earlier. The cells for transplantation were harvested from the spleens and lymph nodes. Because of the data obtained by Volkert and Larsen (13), who showed that the maximal effect could be obtained by transplanting cell doses of about 100×10^6 cells and that the minimal effective dose was about $10–20 \times 10^6$ immune cells, these doses were chosen for our purpose. 100×10^6 cells were employed in three experiments, 20×10^6 in two experiments, and 10×10^6 in one experiment. In order to ascertain the cell-dose dependency of the cell-mediated immune response, doses of 100 and 10×10^6 were used in the same experiment. In all experiments groups of six virus carriers were transplanted intravenously at different times before the cytotoxicity experiment was carried out. On the day of the experiment the spleen and lymph node cells from each group of recipient mice were harvested and

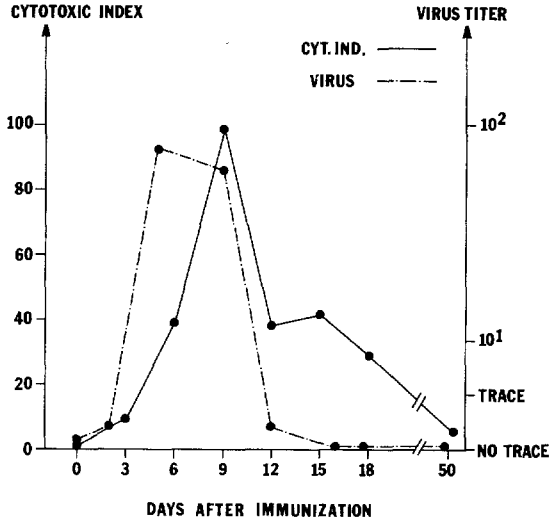

FIG. 3. Cell-mediated immunity and blood virus titers in normal adult C3H mice after intraperitoneal injection of 10^3 LD_{50} of LCM virus on day 0. The cell-mediated immune response is expressed as the cytotoxic indices (CYT. IND.) of lymphoid cells from the different groups of mice. Blood virus titers (VIRUS) are mean titers of eight mice tested with intervals throughout the period of experimentation. (NO TRACE: virus titer $\leqq 10^{0.5}$.)

tested separately for cytotoxicity on target cells from the same batch and in the same culture medium. In addition, the blood was tested for virus, and the serum for complement-fixing antibodies. A pool of lymphoid cells from normal adult mice infected with 10^3 LD_{50} LCM virus 9 days earlier served as a positive control in all experiments.

The results of the three experiments in which 100×10^6 cells had been transplanted were very similar to one another (Table II). The data from one of them are presented graphically in Fig. 4. It appears that a cell-mediated immunity also develops in adoptively immunized virus carriers. This immunity reaches its peak on day 9 and thereafter it declines quite rapidly. Thus this time-course is similar to that described in the acutely infected mice. It is very striking,

however, that the peak value is considerably lower during adoptive immunization. In all the experiments in this series the CIs were below 45, i.e., about half of the value obtained with control cells from acutely infected mice. The experiments in which 10 or 20 $\times$ 10^6 immune cells had been transplanted were also similar to one another. The results are recorded in Fig. 4 and Table II.

TABLE II

The Time-Course of the Cell-Mediated Immunity in Virus Carriers after Intravenous Transplantation of Lymphoid Cells from Immunized Syngeneic Mice

Cell dose	Cytotoxicity of lymphoid cells from recipients expressed as CI						
	Days after transplantation						
	0	2	5	9	12	19	30
100 $\times$ 10^6	0	0	31	37	20	19	11
100 $\times$ 10^6	0	0	0	31	11	6	12
100 $\times$ 10^{6*}	0			40		9	
20 $\times$ 10^6	0	3	3	3	9	7	5
20 $\times$ 10^6	0	0	0	10	14	0	4
10 $\times$ 10^{6*}	0			13		5	

* These results were obtained in one experiment.

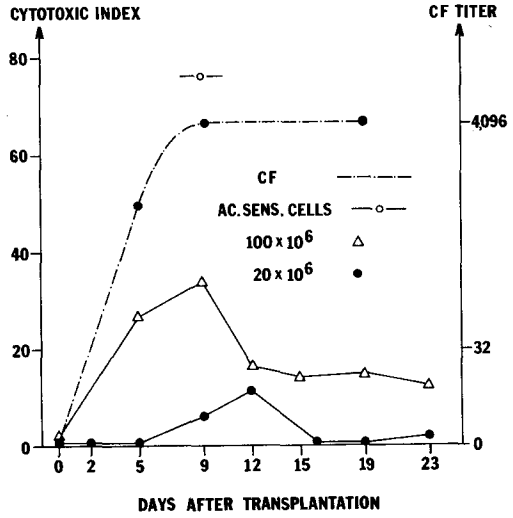

FIG. 4. Complement-fixing antibody titers and cell-mediated immunity in virus carriers adoptively immunized with spleen and lymph node cells from immune syngeneic animals. The complement-fixing antibody titers (CF TITER) are determined on serum pools from six mice, which had received 100 $\times$ 10^6 sensitized cells on day 0. The cell-mediated immune response is expressed as cytotoxic indices of lymphoid cells from virus carriers, which received 100 and 20 $\times$ 10^6 sensitized cells, respectively. Spleen and lymph node cells from normal adult C3H mice injected with 10^3 LD_{50} of LCM virus 9 days earlier (AC. SENS. CELLS) served as positive controls in the cytotoxicity experiments.

When compared with the results just described it is apparent that the cytotoxic activity appeared at about the same time but reached lower values. This was confirmed by the results from the experiments in which the transplantation of 100 and 10×10^6 cells were tested simultaneously (Table II). Fig. 4 also shows the complement-fixing antibody titers obtained when 100×10^6 immune lymphoid cells are transplanted to virus carriers. The results are in complete agreement with the results reported by Volkert and Larsen. It is seen that the titers obtained are about 100 times as high as those seen after an acute infection in normal mice. Moreover, the peak titer is reached just as rapidly as was the case for the cell-mediated immunity. The virus blood titer graphs are not recorded. In all cases the data obtained were in complete accordance with the original findings of Volkert, Larsen, and Pfau (8). The virus disappeared from the blood within 10 days of the transplantation, i.e., during the period in which the rise in both cellular and humoral immunity took place.

The Search for Cell-Mediated Immunity and Enhancing Antibodies in Virus Carrier Mice.—Most of the workers concerned agree that the persistence of high titers of LCM virus in blood and organs in mice infected *in utero* or at birth is due to a state of tolerance to the virus. However, some doubt has also been expressed about the validity of this hypothesis and therefore experiments were carried out to investigate the possibility of the presence of cell-mediated immunity and of enhancing antibodies in these mice.

In the first group of experiments the cytotoxic activity of lymphoid cells from virus carriers and from normal mice was compared. Three experiments were carried out. In each of these lymphoid cell suspensions from the spleen and the lymph nodes were prepared individually from six virus carriers and from six normal mice of the same strain, age, and sex. These cell suspensions were tested separately but simultaneously on target cells from the same batch of infected L cells and in the same culture media. The results from all three experiments agreed very closely and the data from one of them are recorded in Table III. It is apparent that the lymphocytes from the virus carrier mice had no more cytolytic effect than cells from normal mice. Thus our experiments did not reveal any cell-mediated immunity to the virus in LCM virus carrier mice.

In an attempt to demonstrate enhancing antibodies in LCM virus carrier mice, pools of serum were prepared from three groups of virus carrier mice and from three groups of normal mice of the same strain, age, and sex.

Each group of mice consisted of 15 animals. On 3 different days, one of the virus carrier serum pools was compared with a pool of normal serum. The technique employed is described above (Materials and Methods), but it should be added that in all the experiments 10 or more Petri dishes with infected target cells were used per serum pool. The effector cells were in all cases spleen and lymph node cells from C3H mice which had been infected with the LCM virus 9 days previously.

The results from these experiments are recorded in Table IV and clearly demonstrate that there is no significant difference between the CIs obtained after pretreatment of the target cells with serum from normal animals or from virus carriers. Thus no trace of enhancing antibodies was found in virus carrier serum.

Dose Effect of Sensitized Lymphocytes.—It is well known that a stimulation of sensitized lymphocytes with antigen causes release of soluble factors which

TABLE III

The Release of Radioisotope from LCM Virus-Infected ^{51}Cr-Labeled Target Cells after Incubation with Spleen and Lymph Node Cells from Virus Carriers or from Normal Mice

Origin of lymphoid cells	Mouse no.	Release of ^{51}Cr	Mean ± SD	CI
		*cpm**		
Virus carriers	1	3,012		
	2	2,863		
	3	2,730	3,015 ± 79	0
	4	2,853		
	5	2,892		
	6	2,770		
Normal mice	1	2,936		
	2	2,865		
	3	2,884	3,032 ± 31	0
	4	2,737		
	5	2,849		
	6	2,989		

* The cells from each mouse were examined in triplicate, and the mean value is shown.

TABLE IV

The Cytotoxic Effect of Acutely Sensitized Lymphocytes upon LCM Virus-Infected L Cells Preincubated with Normal Mouse Serum or Serum from Virus Carriers

Source of serum	Serum pool*	Release of ^{51}Cr	CI
		cpm	
Normal mice	I	13,888 ± 838	75.3
Virus carriers	I	13,178 ± 624	68.7
Normal mice	II	10,940 ± 278	49.2
Virus carriers	II	11,223 ± 264	51.7
Normal mice	III	37,073 ± 1,530	45.9
Virus carriers	III	38,049 ± 1,320	49.2

* Each pool consisted of serum from about 15 mice.

can influence nonsensitized cells (14). In order to elucidate whether such factors play a role in the cytotoxic test system employed in the experiments described above, a series of experiments was carried out to evaluate the effect of varying numbers of sensitized lymphocytes.

In order to keep the total number of lymphoid cells constant throughout the experiments, mixtures of sensitized and nonsensitized lymphocytes were employed. The sensitized lymphoid cells were prepared from adult C3H mice inoculated i.p. with 10^3 LD_{50} LCM virus 9 days earlier. The nonsensitized cells were from normal mice of the same strain, age, and sex. The mixtures for the experiments were all suspensions containing increasing numbers of sensitized cells and decreasing numbers of nonsensitized cells. Three experiments were carried out, and in each, 11 lymphocyte mixtures containing from 0 to 100% sensitized cells were assayed simultaneously for cytotoxic activity against LCM virus-infected target cells. The final ratio between lymphocytes and target cells was 25:1 in exps. I and II, and 15:1 in exp. III.

The results from all three experiments were very similar and the data are recorded in Fig. 5 and Table V. In the figure the CIs are plotted against the

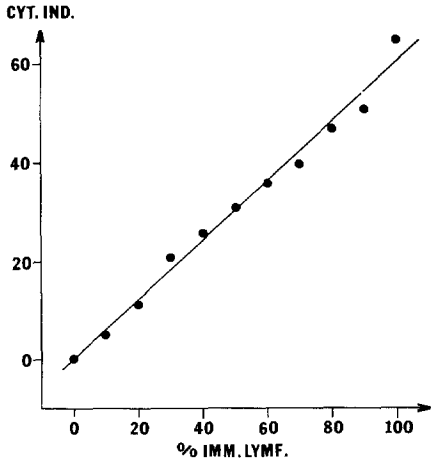

FIG. 5. The cytotoxic effect of different lymphocyte mixtures containing increasing percentages of sensitized cells. The mixtures were prepared from spleen and lymph node cells from C3H mice infected 9 days earlier and from uninfected animals. The ratio between target cells and lymphocytes was 1:25.

TABLE V

The Cytotoxic Effect of Lymphoid Cell Suspensions with an Increasing Ratio between Sensitized and Nonsensitized Cells

Exp.	Cytotoxicity expressed in CI									
	Percentage of sensitized cells in the suspension									
	10	20	30	40	50	60	70	80	90	100
I	29	49	60	67	75	79	80	78	81	79
II	7	24	39	63	78	89	99	99	100	100
III*	5	11	21	26	31	36	40	47	51	65

* This experiment was carried out with a ratio between lymphocytes and target cells of 15:1.

percentage of sensitized cells in the reaction mixtures. It is seen that there is a direct linear relationship between these variables, indicating that the cytotoxic effect is caused by a direct cell-to-cell reaction. Moreover, in the first two experiments a mixture containing about 60% sensitized cells (i.e., 15 sensitized lymphocytes per target cell) was sufficient to cause a maximal effect. In the third experiment, however, in which a final ratio of 15:1 was used, the maximal effect was only just reached, if attained at all.

DISCUSSION

The role of the cell-mediated immune reactions in the pathogenesis of the LCM virus infection in mice is a very intriguing problem. This is mainly due to the fact that it has only recently been possible to demonstrate cell-mediated immune reactions to the LCM virus in vitro, and methods for quantitative in vitro measures have not been available. Such a method has been developed in this laboratory. With the technique described it has been possible to demonstrate the time-course for the cell-mediated immunity during the acute infection in mice. It was very striking that this cell-mediated immunity to the virus followed a course that was similar to the immune responses seen in transplantation immunity experiments (15, 16). In both cases, there is a distinct maximal effect on about the 9th day, followed by a relatively steep decrease to a very low level. The time-course of the D-type hypersensitivity to the LCM virus has also been measured in vivo, using the footpad method, by Tosolini and Mims (2). Their results roughly agree with ours. This indicates that the data obtained in the in vitro test for the viral cell-mediated immunity portray the events which have taken place in the mouse.

As pointed out by Volkert and Lundstedt (1), many experimental data have previously been presented supporting the assumption that it is the cell-mediated immune reaction which eliminates the virus during an acute LCM virus infection. In this paper (Figs. 1 and 2), it is clearly seen that there is a much closer parallelism between the blood virus titers and the cell-mediated immunity than between the virus titers and the development of complement-fixing antibodies. With a delay of about 3 days, the rise and fall of the cell-mediated immunity closely follow the virus titer curve, whereas the antibodies begin to appear so late that by that time the main part of the virus has already been eliminated from the blood. These new data therefore add further support to the view that it is the cell-mediated immunity which is the most important factor in virus elimination.

Experimental data have also been presented (1, 17) indicating that the cell-mediated immunity operates not only during the acute phase of the infection, but that it is also the main factor in keeping an LCM infection on its latent state. Such latent infections very often develop after an ordinary acute LCM virus infection in adult mice, and the animals will usually carry the occult virus for life (6). To keep this virus in check, it would be expected, therefore, that the cell-mediated immunity would continue to operate at a high rate for a long

time after the primary infection. Moreover, the persistance of virus should cause a long-lasting antigenic stimulus which should be sufficient to keep the immunity at a high level. In spite of this our data apparently indicate that the cell-mediated immunity to the LCM virus is of short duration. This is rather surprising. On the other hand it resembles the situation seen in mice after an intravenous stimulation with sheep red blood cells. In these animals there is a short lag phase, after which the number of antibody-producing cells in the spleen increases exponentially for 2–3 days and then declines to a low level (18). Why and in what way the immunologically active cells disappear is not known, but they may either have a short life-span, be inactivated by inhibitors of some kind, or perhaps just migrate out of the spleen and the lymph nodes. With regard to the last possibility, Tosolini and Mims' experiments, mentioned above, strongly indicate that even in the entire animal the cell-mediated immunity (expressed as D-type hypersensitivity) disappears shortly after the peak of the LCM viral infection. The disappearance of the active cells seems, therefore, to be more than a local phenomenon.

The importance of the cell-mediated immunity for the viral elimination which takes place during an adoptive immunization of neonatally infected virus carriers is uncertain. In these cases, no significant time difference between the humoral and the cell-mediated immune responses could be demonstrated. Moreover, the intensity of the cell-mediated immunity was markedly lower than the response during the acute infection, whereas the antibody titers could reach values about 100 times as high as those obtained in acutely infected mice. When these results are considered together with the fact that adoptively immunized virus carrier mice also develop antibodies with a strong virus-neutralizing capacity, a phenomenon never seen in mice acutely infected with the LCM virus, we are inclined to believe the humoral immunity to be the most important factor in the elimination of the virus in adoptively immunized mice.

For many years it has been widely accepted that a state of tolerance to the LCM virus could be established in mice. However, Oldstone and Dixon (19) have recently published data indicating that some degree of humoral as well as cell-mediated immunity may be present in the apparently tolerant mice. Moreover, Allison (personal communication) has suggested that in such mice enhancing antibodies may obscure the presence of cell-mediated immunity. In our experiments these hypotheses were put to a direct test. The experiments revealed, however, that the cytotoxic effect of lymphocytes from virus carriers did not exceed the effect of lymphocytes from normal mice and, moreover, serum from virus carrier mice was not able to protect target cells from the lytic effect of activated lymphocytes from acutely infected mice. Thus our experiments failed to produce any evidence indicating either cell-mediated immunity to the virus, or the presence of enhancing antibodies in the virus carrier mice. However, as might be the case in ordinary adult mice a long time after an acute infection, the active cells, if present in virus carriers, might not be located in the

organs used for cell preparations. Furthermore the enhancing antibodies, if present in the serum from virus carriers, might not be able to cover all the virus-induced cell surface antigens which are continuously produced during the 18 h duration of the in vitro cytotoxic test. However, because of the fact that preliminary experiments carried out in this laboratory have revealed that the target cells were not protected even when the serum from carrier mice was present during the entire cytotoxic assay, this assumption does not seem very likely. We can only conclude that our results do not completely eliminate the possibility of the presence of cell-mediated immunity in virus carriers, but they do not, on the other hand, provide any support for the hypothesis.

In recent years controversial hypotheses concerning the basic mechanism of the in vitro cytotoxic reaction have appeared. It has been claimed that a soluble factor released from sensitized lymphocytes after contact with antigen might be the mediator of the cytolytic reaction (20, 21). Moreover, Oldstone and Dixon have demonstrated the existence of a soluble cytolytic factor released after several days of incubation of immune lymphoid cells with the LCM virus (22). In Oldstone and Dixon's experiments, the release of the factor seems to be immunologically specific, but once released the cytolytic effect seems to be nonspecific.

Other investigators have searched for soluble factors which might play a role in the lymphoid cytotoxic reaction, but have failed to demonstrate any such factors, and it seems that the majority of the workers concerned are inclined to believe that the cytolysis of target cells brought about by sensitized lymphocytes is caused by a direct cell-to-cell contact (23, 24). The results from our dose response experiments reveal a direct linear relationship between the number of sensitized lymphocytes and the degree of cytolysis. This supports the assumption that a possible soluble factor taking part in this reaction cannot be of major importance. Moreover, the "one-hit kinetic" type of this curve indicates that only one type of lymphoid cells is necessary to bring about the destruction of a target cell.

SUMMARY

A large amount of experimental evidence has already been presented indicating the great importance of the cell-mediated immunity in the pathogenesis of the LCM virus infection in mice. In this laboratory a method which makes it possible to measure this cellular immunity quantitatively in vitro has been developed. The method is based on the determination of the radioisotope released after the interaction between specifically sensitized lymphocytes and syngeneic ^{51}Cr-labeled LCM virus-infected target cells. By using this technique the time-course of the cell-mediated immunity has been established in acutely infected mice and in virus carriers adoptively immunized with syngeneic sensitized lymphocytes. Lymphocytes from acutely infected mice showed a strong lysing effect on the target cells, with a sharp maximum at about the 9th day after in-

fection. The cell-mediated immunity in adoptively immunized virus carrier mice showed the same time-course, but in these animals the lytic effect of the lymphoid cells was considerably less pronounced. Lymphocytes from untreated virus carriers did not, however, have any effect on the target cells, and in these animals it was not possible to demonstrate any evidence of enhancing antibodies. In experiments employing serial dilutions of sensitized lymphocytes in normal cells a direct linear relationship between the number of sensitized lymphocytes and target cell destruction was found. These experiments seem to indicate that the underlying mechanism in the cytotoxic reaction is a direct cell-to-cell interaction.

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